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SEASONAL RELIABILITY OF TESTOSTERONE RADIOIMMUNOASSAY (RIA) FOR PREDICTING SEX RATIOS OF JUVENILE LOGGERHEAD (*CARETTA CARETTA*) TURTLES

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ABSTRACT: Because sex is determined by incubation temperatures in sea turtles and immature animals are not sexually dimorphic externally, circulating levels of testosterone measured with radioimmunoassay (RIA), in conjunction with laparoscopies, have been used to estimate sex ratios. From September to December 1995 to 1997, and from June to December 1998 to 2002, we sampled blood from 1106 juvenile loggerhead sea turtles (*Caretta caretta*) incidentally captured in pound nets set in Core and Pamlico Sounds, North Carolina to measure testosterone levels. Laparoscopies of 89 of these turtles revealed a sex ratio of 2.1F:1M, similar to other juvenile loggerhead populations along the southeastern coast of the USA. Laparoscopies demonstrated that testosterone levels correctly identified males during summer months (water temperatures >23 C), but were unreliable during late autumn/winter months (water temperatures ≤16 C). During the summer months, females (n = 201) exhibited testosterone concentrations with an upper limit of 239.0 pg/ml, and males (n = 69) exhibited a lower limit of 372.0 pg/ml, for a sex ratio of 2.9F:1.0M. We recommend that verification of the RIA should be conducted by laparoscoping a subset of turtles sampled in all sex ratio studies. In addition, this verification should be conducted at several different times throughout the year to evaluate any possible seasonal effects on testosterone concentrations.

Key words: Caretta caretta; Laparoscopy; Loggerhead sea turtle; Radioimmunoassay; Sex ratio; Testosterone

LIKE many reptile species, the temperature at which sea turtle eggs are incubated determines the sex of the hatchlings, a process known as temperature-dependent sex determination (TSD) (reviewed by Janzen and Paukstis, 1991). This lack of heteromorphic sex chromosomes combined with the absence of dimorphic secondary traits in juveniles can make it challenging to determine the sex ratio within a population. To add to the difficulty, apparent sex ratios may vary depending on the life stage being studied (Wibbels et al., 1987*b*; Wibbels et al., 1991). For example, the sex ratios of hatchling populations can vary from year to year depending upon the geographic location of nests, time of year the eggs are laid, and annual weather patterns (Mrosovsky, 1994; Mrosovsky et al., 1984). Ascertaining adult sex ratios can be problematic because various sex-specific behaviors (i.e., mating, remigration interval) make it difficult to randomly sample the adult population (Wibbels et al., 1987*b*; Wibbels et al., 1991).

With all of the difficulties inherent in studying hatchlings and adults, it is possible that population sex ratios might be assessed more accurately by evaluating neritic juveniles (Shoop et al., 1998; Wibbels et al., 1987*b*). Because of the long duration of the neritic life stage, it represents many cohorts and is therefore a condensation of many years of hatchling production, integrating sex ratio variability over time (Wibbels et al., 1987*b*; Wibbels et al., 1991). In addition, neritic juveniles are less likely to have developed the sex-specific behavioral biases exhibited by adults. As a result, studies of this stage are more likely to reveal the actual secondary sex ratio for a given population, (Shoop et al., 1998; Wibbels et al., 1987*b*), which, in turn, is a better indicator of the population's future reproductive potential (Wibbels et al., 2000).

Several sex determination techniques for sea turtles are available to researchers (Wibbels, 1999). Although laparoscopy is an

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accurate method for sexing juvenile sea turtles (Wibbels et al., 2000; Wood et al., 1983), it is logistically difficult. Because laparoscopy is an invasive surgical procedure, it is difficult to perform in the field, and relatively large amounts of time and effort must be spent on each turtle (Wibbels et al., 2000), potentially limiting the number of turtles that can be sampled. In contrast, plasma testosterone concentration has been determined to be an accurate indicator of the sex of juvenile sea turtles (see Owens, 1997 for a review) and only involves collection of blood samples, which is far less invasive than laparoscopic procedures. Because of the ease with which blood can be drawn, a greater number of turtles can be sampled using this technique, and therefore testosterone radioimmunoassay (RIA) (Owens et al., 1978) appears to be the most practical means to successfully sex large numbers of juvenile sea turtles (Wibbels, 1999, 2003). However, given that testosterone levels in individual juvenile sea turtles may vary seasonally (Gregory et al., 1996; Morris, 1982; Owens, 1997), RIA should be validated with a sub-sample of turtles whose sex was verified using laparoscopy (Wibbels et al., 2000).

We investigated the sex ratio of a population of juvenile loggerhead (*Caretta caretta*) sea turtles inhabiting Pamlico and Core Sounds, North Carolina, U.S.A as part of an on-going, long-term study to assess populations of sea turtles along the U.S. Atlantic coast (Epperly and Braun, 1998). We used plasma testosterone RIA and confirmed the sex of a subset of the sampled turtles through a laparoscopic examination of their gonads. Our results demonstrate that although RIA can reliably predict turtle sex during the summer months, this technique may not be as reliable during late autumn/winter.

Materials and Methods

Study Site and Capture Technique

From September to December 1995 to 1997, and from June to December 1998 to 2002, we obtained loggerhead turtles from pound nets set in Core and Pamlico Sounds, North Carolina, U.S.A. (Fig. 1). Pound nets are a type of passive, stationary fishing gear

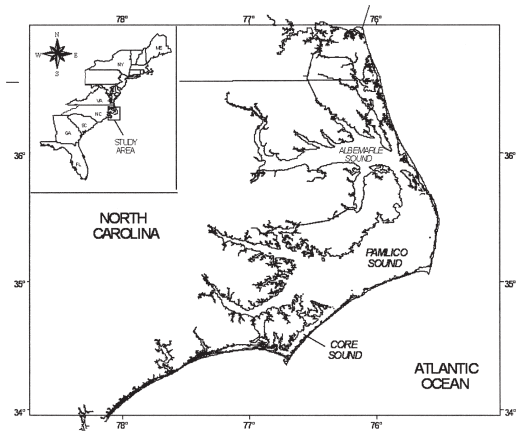

Fig. 1.—Juvenile loggerheads (*Caretta caretta*) were captured in pound nets set in Core and Pamlico Sounds, North Carolina, U.S.A.

that incidentally captures turtles but allows them to surface and breathe (Higgins and Pearson, 1928). We collected blood samples from each turtle within 30 min after removal from the pound net. We excluded turtles with a carapace length greater than 76 cm from the analysis to prevent the use of adults in this study (and avoid the bias associated with various sex-specific behaviors of the adult population) (Wibbels et al., 1987*b*; Wibbels et al., 1991). For each turtle, we measured standard straight-line carapace length (SCL) to the nearest 0.1 cm using calipers. We recorded surface water temperatures to the nearest 0.5 C using calibrated thermometers.

Sexing Technique

We collected 5 ml of blood from the dorsocervical sinus of each turtle (Owens and Ruiz, 1980) using a sterile syringe with a 3.81 cm, 20 gauge needle into a sterile lithium heparin or sodium heparin blood collection tube and immediately stored it on ice for a maximum of 5 h (i.e., for the rest of the field day). We centrifuged blood samples for 6 min, then pipetted 2 ml samples of plasma from each turtle into cryogenic vials and froze them at −80 C. A plasma androgen sexing technique was used to classify sex of the turtles using a testosterone radioimmunoassay (RIA) procedure (Owens, 1997; Owens and Hendrickson, 1978; Wibbels et al., 1987*b*). A

recently discovered technical error in the RIA (due to a mislabeled testosterone standard used for many years) has resulted in testosterone levels in this study that are an order of magnitude higher (i.e., correction factor of 10×) than what had been previously reported by the Owens collaborators (for example Owens, 1997; Wibbels et al., 1987*b*) (Lee, 2003). A thorough laboratory investigation has shown that the precision of the assay has remained unchanged and is acceptable (Lee, 2003). Although the results of this study are not affected by the technical error, it is a factor to consider when making comparisons with results of previous studies.

To validate sex classification using RIA, we first determined sex for a sub-sample of turtles via laparoscopy in November 1997, August 2000, and July 2001. Laparoscopy is an effective, accurate method of evaluating the sex of juvenile sea turtles (Wibbels, 1999; Wood et al., 1983). Visual classification of the ovary and testis was based on examination of the surface of the gonads: the ovaries have a regular undulating appearance caused by the underlying primordial follicles in contrast to the seminiferous tubules underlying the smoother stroma of the testis. We then used testosterone levels of known sex turtles (i.e., those that underwent laparoscopy) to estimate the plasma testosterone range for each sex and to predict the sex of the rest of the turtles (Wibbels et al., 2000).

We used the score-test based method for computing binomial confidence intervals (Agresti and Coull, 1998) to determine if there was a significant difference in sex ratio for each 10 cm size class.

Results

We sampled blood from 1106 loggerhead turtles with carapace lengths ranging from 41.4–75.7 cm and conducted laparoscopies for 89 of them. Plasma testosterone concentrations ranged from 0.1–6177.0 pg/ml. The sex ratio of turtles subject to laparoscopies was 2.1F:1.0M. Plasma testosterone concentrations for the laparoscoped females ranged from 6.7–147.0 pg/m ($n = 60$); the laparoscoped males ranged from 61.0–1884.0 pg/ml ($n = 29$). Testosterone concentrations of 25 of these turtles overlapped in the zone of 61.0 and 147.0 pg/ml; therefore, we were unable to establish threshold levels of plasma testosterone for males and females from these data alone.

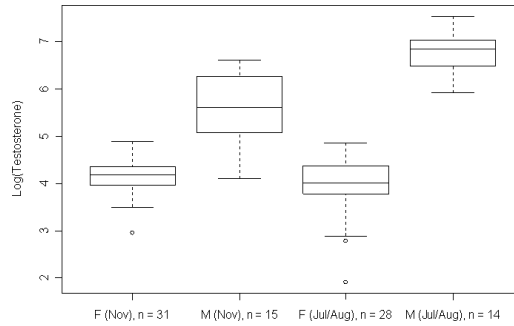

FIG. 2.—Log-transformed plasma testosterone concentration, with sample size (n), from laparoscopically examined male (M) and female (F) loggerhead (*Caretta caretta*) turtles collected during late autumn/early winter months (November) and summer months (July/August). Whiskers represent 95% confidence interval and box represents the interquartile range.

To evaluate a possible seasonal effect on testosterone concentrations (Gregory et al., 1996; Morris, 1982; Owens, 1997) and to establish threshold levels of plasma testosterone for males and females, we first compared the testosterone concentrations of loggerheads subject to laparoscopies in November (water temperatures: 14–16 C) with those that underwent laparoscopies in July and August (water temperatures: 24–28 C). Turtles that were laparoscoped during November (2.1F:1.0M) had testosterone concentrations that overlapped (females: 19.0–147.0 pg/ml, $n = 32$; males: 61.0–737.0 pg/ml, $n = 15$). In contrast, those that underwent laparoscopies during July and August (2.0F:1.0M) had testosterone concentrations that did not overlap (females: 6.7–128.0 pg/ml, $n = 28$; males: 372.0–1884.0 pg/ml, $n = 14$) (Fig. 2). Because of the lack of overlap in the latter case, we were confident of the predictive capabilities of the RIA for sexing loggerhead turtles whose blood was sampled during July and August. In addition, we noted a significant positive relationship ($P < 0.001$) between temperature ($y = 0.07x + 2.96$; $r^2 = 0.10$) and photoperiod ($y = 0.21x + 1.83$; $r^2 = 0.08$) with log-transformed testosterone concentrations, suggesting a seasonal effect (Fig. 3). Photoperiod

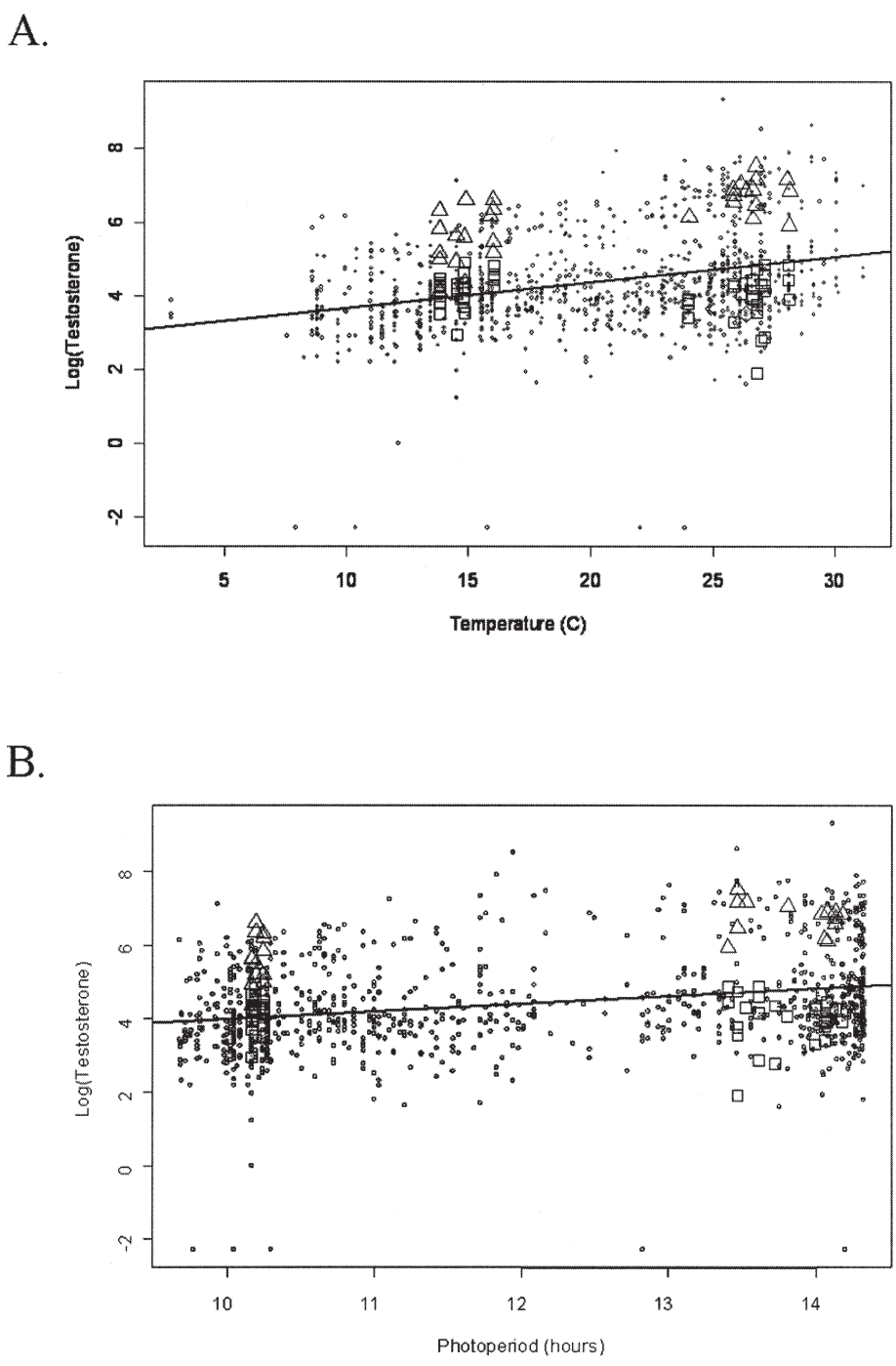

FIG. 3.—Log-transformed plasma testosterone concentration of all loggerhead (*Caretta caretta*) sea turtles sampled at varying (A) water temperatures ($y = 0.07x + 2.96$; $r^2 = 0.10$; P-value < 0.001) and (B) photoperiods ($y = 0.21x + 1.83$; $r^2 = 0.08$; P-value < 0.001). Triangles and circles indicate laparoscopic sex determinations for males and females, respectively. Plasma testosterone concentrations ranged from 0.1–6177.0 pg/ml.

was approximated using a simple geometric relationship of the earth-sun system applied to latitude of 34.75 °N (Fig. 1).

To compare sex ratios within size classes, we established threshold levels for females and males by using the highest female testosterone concentration (128.0 pg/ml) and the lowest male testosterone concentration (372.0 pg/ml) obtained from turtles subject to laparoscopies during July and August (water temperatures 24–28 C). Two of the females laparoscoped had been sampled for testosterone during July and August in previous years, allowing us to extend the upper level of the testosterone range for females. For example, the testosterone concentration of CC-1 when sampled in August 1999 (water temperature = 28 C) was 205.0 pg/ml; the testosterone concentration of CC-2 when sampled in July 2000 (water temperature = 26 C) was 239.0 pg/ml. Thus, we were able to extend the testosterone range for females from 128.0 pg/ml to 239.0 pg/ml. Since small sample sizes prevented us from comparing sex ratios of turtles that underwent laparoscopy for each 10 cm size class, we compared sex ratios of turtles whose sex was estimated using testosterone concentrations instead. We applied testosterone threshold levels to testosterone samples collected during July and August (water temperatures 21–31 C; the time period during which we were confident of the predictive capabilities of the RIA for sexing loggerheads) (Fig. 4) and generated an overall sex ratio of 2.9F:1.0M. We determined that there was no significant difference in sex ratio among the size classes at the 95% confidence level (Table 1). Unknowns were not included in the calculation of the sex ratio.

All 14 of the males subject to laparoscopies during July and August had testosterone levels >372.0 pg/ml while 10 of the 15 males that were laparoscoped during November had depressed testosterone concentrations (<372.0 pg/ml); based on the criteria we developed above, 7 of these 10 would have been classified as female (<239.0 pg/ml), the other 3 as unknown (>239.0 pg/ml but <372.0 pg/ml). Also, a male whose sex was later verified via necropsy had a depressed testosterone level (72.9 pg/ml) at initial capture; this sample was collected

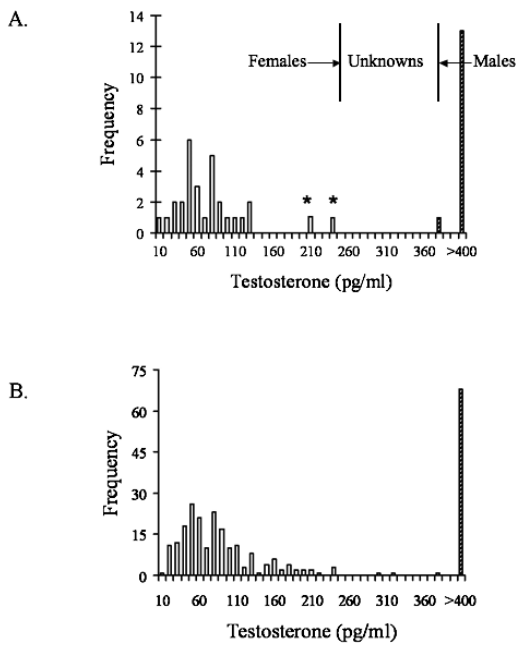

FIG. 4.—Frequency of plasma testosterone concentration of juvenile loggerhead (*Caretta caretta*) turtles sampled during July and August (A) that were laparoscopically examined (n = 89) (water temperatures 24–28 C) and (B) that were not laparoscopically examined (n = 270) (water temperatures 21–31 C). Vertical lines indicate threshold testosterone titer of 239.0 pg/ml for females and 372.0 pg/ml for males. Clear bars indicate females; striped bars indicate males. Asterisks (*) denote the testosterone concentration of females we had conducted laparoscopies on and which were sampled multiple times.

November 1998. Had we relied on testosterone concentration alone, we would have misclassified the sex of these turtles. In addition, three turtles (whose sex was not verified by laparoscopy) sampled during the year at varying water temperatures had testosterone levels that varied with the season (Table 2). Because these levels were above the threshold for males during the summer (>372.0 pg/ml), we assumed they were males. Had these turtles been sampled only during late autumn/winter months, they would have been misclassified as females based on their testosterone concentration.

DISCUSSION

The Endangered Species Act (1973) requires the establishment of management plans

TABLE 1.—Testosterone concentration (pg/ml) and predicted sex of three loggerhead (*Caretta caretta*) turtles sampled at varying water temperatures (C). Testosterone concentration ≤255.0 pg/ml would result in a female classification.

Turtle ID	Date	Water temperature (C)	Testosterone concentration (pg/ml)	Sex as determined by testosterone concentration
CC-1	23 September 2002	25.5	776.0	M
	5 November 2002	17.4	56.0	F
CC-2	3 August 1998	22.5	480.0	M
	9 November 1998	13.5	115.0	F
	21 June 1999	23.1	1260.0	M
CC-3	13 November 2001	12.1	141.0	F
	8 July 2002	24.9	1642.0	M
	31 August 2002	27.1	512.0	M

that will effectively provide for the protection and recovery of sea turtle populations listed as threatened and endangered. Information about population size and the status of sea turtle stocks is essential for conservation biologists to formulate recovery actions, as well as to monitor recovery. Such information historically has come from nesting beach surveys (National Research Council, 1990). However, given that sea turtles are slow to mature, a population's response to recovery actions cannot be observed on nesting beaches until many years after those actions are initiated (Bjorndal et al., 1999). Therefore, a better way to measure the success of management actions is by monitoring in-water juvenile populations, which would reflect responses to these actions more rapidly than adult populations (Wibbels et al., 1991).

Monitoring efforts should not be limited to estimates of population size, but also should include the demographic characteristics of the population, including age, stage, stock, and sex composition (Casale et al., 1998; Gerrodette and Gilmartin, 1990). The sex ratio (relative proportion of females and males) is of particular interest, as it can potentially affect population dynamics (e.g., intrasexual competition, fecundity, or time required to find a mate) (Gibbons, 1990). In addition, significant deviations from a natural sex ratio can indicate potential problems within a population (Janzen, 1994; Meffe and Carroll, 1997). Skewed population sex ratios are of special concern for animal populations exhibiting temperature-dependent sex determination (TSD), as even moderate climatic changes (i.e., global warming) can significantly affect this demographic parameter (Janzen, 1994).

TABLE 2.—Estimated sex ratios and sample size (N) for each size class of loggerhead (*Caretta caretta*) turtles whose sex was classified from testosterone samples collected during July and August. Differences were not significant at the 95% confidence level.

Size class (SCL in cm)	Estimated sex ratio	Sample zize (N)	95% confidence interval
40–49.9	2.0F:1.0M	15	0.7:1–5.6:1
50–59.9	3.7F:1.0M	103	2.3:1–5.9:1
60–69.9	2.4F:1.0M	114	1.6:1–3.7:1
70–75.0	3.0F:1.0M	36	1.4:1–6.3:1

During this study, we found that the sex ratio of juvenile loggerhead sea turtles inhabiting North Carolina inshore waters is female-biased whether derived from laparoscopies (2.1F:1.0M) or from testosterone levels of turtles sampled during the summer months (2.9F:1.0M). Furthermore, we were unable to classify the sex of only two turtles (<1%); thus classifying these two individuals as either male or female would have no effect on the resulting sex ratio of our population. Similar female biases also have been found in other sex ratio studies of juvenile loggerhead sea turtles to date: Chesapeake Bay, Virginia (2.0F:1.0M) (Wibbels et al., 1987*b*), Cumberland Island, Georgia (1.9F:1.0M) (Shoop et al., 1998), and Cape Canaveral (1.7F:1.0M) (Wibbels et al., 1987*b*), Hutchinson Island (2.5F:1.0M) (Wibbels et al., 1987*a*; Wibbels et al., 1991), and Indian River, Florida, (1.4F:1.0M) (Wibbels et al., 1987*b*) (summarized by Owens, 1997). In addition to these studies involving live turtles, the sex ratios of dead-stranded loggerheads exhibited a comparable bias toward females: Virginia (2.1F:1M), North Carolina (1.9F:1M), South Carolina (2.1F:1M), Georgia (1.7F:1M), and Florida (1.9F:1M) (National Marine Fish-

eries Service Southeast Fisheries Science Center, 2001).

If the female-biased sex ratio (about 2F:1M according to Owens, 1997) of the juvenile population is representative of the adult loggerhead sea turtle population along the southeast coast of the United States, this could have important implications for conservation biologists managing loggerhead populations. Previous population models used to assess the status of loggerhead sea turtles have incorporated 1:1 as the default sex ratio (Crouse et al., 1987; Crowder et al., 1994; Heppell et al., 2003*a*). However, in a recent stock assessment of the loggerhead sea turtle (National Marine Fisheries Service Southeast Fisheries Science Center, 2001), population models that considered a predominately female population (80% female) displayed higher population growth rates than those based on populations that were 1:1 or predominately male. Thus, in order for accurate population assessments to be made, the sex ratio of the population needs to be determined.

Juvenile sea turtle populations, such as the one in this study, comprise individuals originating from several genetically distinct nesting subpopulations (Heppell et al., 2003*b*), each of which potentially has a different sex ratio (Mrosovsky and Provancha, 1992). Those beaches experiencing relatively higher incubation temperatures can produce a higher proportion of females when compared to those beaches having lower incubation temperatures. For Atlantic loggerheads, it is estimated that Florida's beaches (contributing to the southern Florida subpopulation) are producing as high as 90–95% female hatchlings (Hanson et al., 1998; Mrosovsky and Provancha, 1992). In contrast, Georgia and South Carolina beaches (contributing to the northern Florida to North Carolina subpopulation) are estimated to produce 50–60% female hatchlings (Mrosovsky et al., 1984); however, no published data currently are available to confirm this. Using natal origin probabilities from three juvenile foraging populations along the southeast Atlantic and Gulf coasts (North Carolina, South Carolina, and Texas), along with observed sex ratio data from each of those sites, NMFS SEFSC (2001) attempted to estimate the primary sex ratio of the contributing natal populations. They found that the northern subpopulation did not exhibit a female bias (35%) while the south Florida (80%) and Yucatan (69%) subpopulations did.

Considering that 90% of Atlantic loggerheads originate from beaches where, presumably, as high as a 9:1 female to male hatchling sex ratio exists (Hanson et al., 1998; Mrosovsky and Provancha, 1992), the approximately 2F:1M sex ratio observed for juvenile loggerheads in the Atlantic (Owens, 1997) seems rather low. Hopkins-Murphy et al. (2003) calculated an expected 6:1 female to male juvenile sex ratio based on hatchling sex ratio data, the relative contribution of each nesting subpopulation (however, they only considered the northern and southern Florida subpopulations, citing incomplete data and small population size of the other subpopulations), and clutch sizes. The expected sex ratio, however, may be even higher than what Hopkins-Murphy et al. (2003) estimated as recent genetic studies (Bass et al., 2004) are revealing a smaller contribution (12%) of the northern subpopulation (where, presumably, a greater percentage of male hatchlings are produced) to the juvenile populations than what had been previously calculated. Nevertheless, these calculated sex ratios are still considerably higher than those observed in juvenile loggerhead sex ratio studies to date.

Hopkins-Murphy et al. (2003) proposed several different hypotheses to explain this apparent discrepancy between primary and secondary sex ratios. One possibility is that the hatchlings from the southern Florida subpopulation experience a greater rate of mortality than hatchlings from the northern subpopulation, or male hatchlings in general. The authors also suggested that other foraging grounds—that have not yet been surveyed—may display the predicted highly female-biased sex ratio. Clearly, further studies are needed before the disparity between the observed juvenile sex ratios and that expected from hatchlings can be explained.

Because juvenile sea turtles lack external sexual characteristics, circulating levels of testosterone often have been used to predict sex. While many studies have used this technique to accurately determine the sex of

relatively large numbers of sea turtles, there are some circumstances (e.g., samples collected September–December of the present study) under which testosterone levels may be misleading if environmental conditions are not considered carefully (Owens, 1997). During early studies of testosterone levels in loggerhead turtles at Cape Canaveral, Florida, Wibbels et al. (1987*b*) noted a striking difference in the seasonal plasma concentration of testosterone for juveniles and adults. Juveniles appeared to show reduced testosterone in winter months while adults showed almost the opposite effect, with an increase in testosterone at the onset of reproduction, starting in the winter when water temperatures are still relatively cold. Owens (1997) has speculated that this disparity might occur due to a developmental switch in sensitivity to different environmental cues; whereas juveniles may respond to temperature, adults may respond more to photoperiod because the large pineal system begins functioning at the reproductive level during puberty.

Another interpretation of the positive relationship between temperature/photoperiod and testosterone concentrations found in this study is that instead of reflecting a change in testosterone levels, it may signify that males left our study area prior to females as water temperatures/photoperiod decreased. However, if this were true, then we would predict that the sex ratio of loggerhead turtles that underwent laparoscopies during the autumn would reflect this behavior, and it does not. The female-biased sex ratio of the turtles that had been subject to laparoscopies during July and August (2.0F:1.0M, $n = 42$) was similar to that of the turtles that underwent laparoscopies during November (2.1F:1.0M, $n = 47$). In addition, the sex ratio of loggerhead turtles stranded dead along North Carolina's coast during November and necropsied was similar to that obtained via laparoscopy (1.8F:1.0M, $n = 22$, SCL $\leq$ 76.0 cm)(North Carolina Wildlife Resources Commission, unpublished data).

The data yielded by this study suggest that testosterone concentrations were depressed in blood samples collected during late autumn/winter months (water temperatures were $\leq$16 C). As a result; it appears that RIA cannot be used reliably to classify sex of juvenile loggerhead sea turtle males in North Carolina estuaries at this time of year. In contrast, testosterone concentrations of turtles that had undergone laparoscopies in July and August were indicative of sex and could therefore be used as a reliable predictor during this time of year (water temperatures 24–28 C). The significant positive relationship of water temperature and photoperiod with testosterone concentration indicates that the expression of testosterone concentration in sea turtles is affected by a seasonal component (water temperature, photoperiod, seasonal food availability, seasonal feeding behavior). Further studies under a controlled setting will allow a determination of each environmental factor's effect on testosterone concentration. We recommend that verification of the RIA should be conducted by laparoscoping a subset of turtles sampled in all sex ratio studies (Wibbels et al., 2000). In addition, this verification should be conducted at several different times throughout the year to evaluate any possible seasonal effects on testosterone concentrations.

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